

PARTICLE INDUCED X-RAY EMISSION STUDY OF
ROD OUTER SEGMENT DISC MEMBRANES

BY

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By

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A relatively new physics technique, particle induced X-ray emission (PIXE), was used to determine the trace element content of bovine retinal disc membranes. In this technique, characteristic X-rays resulting from inner shell atomic vacancy production by heavy, charged particles are detected. With standardization the amount of each element in a sample can be determined. PIXE is multi-elemental allowing the simultaneous determination of all trace elements and making it a very useful biological technique. The multi-elemental property of PIXE was used in this study to determine the trace element content per rhodopsin without performing a separate rhodopsin assay. First, the ratio of each element to sulfur in a sample was determined. This ratio was then related to the amount of rhodopsin using a sulfur content of ten per rhodopsin. Disc membranes were obtained by a standard separation procedure.

The amount of trace elements per rhodopsin were as follows: calcium $.492 \pm .153$, iron $.0243 \pm .0075$, copper $.132 \pm .060$, and zinc $.0577 \pm .010$. In addition chlorine was present at a level of $.903 \pm .445$ per rhodopsin. Commonly occurring biological metals which were not detected are manganese, molybdenum and nickel. Upper limits determined per rhodopsin for these elements are .007, .0060 and .0033 respectively. No concentration changes of any of the detected elements were noted after light exposure of disc membrane samples. Bleaching levels used were between .1% and 100%.

CHAPTER I

INTRODUCTION

Sensory receptors are highly specialized cells which capture energy and initiate an impulse in adjoining nerve cells. This process is referred to as transduction. For each type of receptor cell, researchers are attempting to isolate and subsequently study the molecule responsible for energy absorption. Concurrent with this they are trying to understand the transduction process.

In the visual process, the transducing molecule has been identified and extensively studied.¹⁻⁴ Rhodopsin is the molecule responsible for absorbing light and beginning the visual chain of events. Vision is unique among the senses, because no other transducing molecules have been discovered.

In 1876, Franz Boll successfully demonstrated the presence of a visual pigment in a freshly isolated retina, and noted that light incident upon the retina caused the characteristic red color to change to white.⁵ This process is referred to as bleaching.

The role of rhodopsin in transduction was the subject of many brilliant studies conducted by Wald and co-workers.⁶⁻⁷ It is largely due to these studies that visual transduction has been characterized on a molecular level. As a result, it has been widely studied by biophysicists attempting to discover the mechanism for the conversion of light energy into a nerve

impulse. This dissertation is the report of one such study of the visual transduction mechanism.

CHAPTER II

RODS, DISCS, AND RHODOPSIN

The posterior portion of vertebrate eyes, the retina, often contain two different types of visual receptor cells, rods and cones.⁵ Each cell is named because of its distinctive shape. Cones are responsible for color vision, whereas, rods are primarily useful at low light intensities. Rods consist of two distinct sections, the inner and outer segment. The inner segment contains organelles which maintain the cell. In contrast, the outer segment consists almost entirely of flattened sacs formed by two parallel disc shaped membranes. Most of the cell's rhodopsin is contained in the disc membranes. Thus, it is expected that this portion of the cell is responsible for transduction.

Rod outer segments are large on a cellular scale. Bovine rod outer segments are approximately 7-10 micrometers in length and 2 micrometers in diameter⁸ resulting in a volume as large as 3×10^{-14} liters. Their large size enables cell physiologists to pierce the external (or plasma) membrane and monitor the transmembrane electrical signals. This type of research has yielded many results.^{2,3,5} Even though these studies cannot determine the mechanism of transduction, any theory attempting to explain transduction must agree with the results obtained.

Using thin section electron microscopy, Mason has

determined a typical disc to disc repeat distance of 250 angstroms and an intradisc space of 25 angstroms. Each disc membrane has a thickness of 75-100 angstroms. The membrane forms a sac and each bovine rod outer segment contains approximately 450 discs.⁹ The dimensions of discs in other vertebrates as determined by electron microscopy and X-ray diffraction are similar.¹⁰ (See Figure 1.)

The sacs are layered or stacked in the rod outer segment and surrounded by the plasma membrane. The disc plane is perpendicular to the rod's longitudinal axis. Electron micrographs indicate there is no contact between the disc and plasma membrane.⁹ It should be noted that electron microscopy requires extensive sample preparation and the results are not always considered conclusive.

The biogenesis of disc membranes is unclear. Mason has indicated that the discs are formed as the result of fusion of small vesicles observed at the base of the rod outer segment near the cilium connecting outer and inner segments.⁸ However, Young and other researchers have noted what he described as invaginations of the outer segment plasma membrane, forming layered discs.¹¹

It is generally agreed that rhodopsin is synthesized in the ribosomes of the inner segment, afterward migrating to the Golgi apparatus. However, the role of the Golgi apparatus in this sequence is unknown.¹² Subsequently, rhodopsin migrates through the connecting cilium and is placed in the discs. The details of rhodopsin synthesis and incorporation into the disc

membranes are currently the object of much research by biochemists and cell physiologists.

After formation, the discs move toward the posterior of the outer segment, eventually being phagocytized by the pigment epithelium. The process typically requires several weeks but varies considerably among species and with the amount of light exposure.

The study of the planar arrangement of discs has been accomplished using low angle X-ray diffraction and neutron scattering. Each technique indicates that rhodopsin is located in each membrane of the disc bilayer and has a relatively uniform cross section which completely spans a membrane.¹³

Raman and circular dichroism studies of disc membranes suggest that most of the protein is alpha-helical, indicating a high degree of organization.^{14,15} This is interpreted by some researchers to imply the molecule forms a "channel" spanning the disc membrane. This is of importance in the currently accepted models of the transduction mechanism.

The biochemical composition of disc membranes is well known and unusual; because, the protein component is dominated by one protein, rhodopsin, which is 87% of the disc protein. The remaining protein components are not as well known. Lipids comprise 50% of the disc membranes. Protein accounts for 36%, and carbohydrates make up 4%.¹⁶

Rhodopsin is a combination of the membrane protein opsin and the chromophore 11-cis retinal. Retinal is probably bound to opsin with an ϵ -nitrogen of lysine.¹⁷ One important unexplained problem of vision is how this bonding yields a

maximum absorption above 500 nanometers instead of 440 nanometers which would be expected based upon similar compounds.⁷

CHAPTER III

IONIC THEORY OF TRANSDUCTION

The excitation process in a vertebrate rod is known to begin with a conformational change in rhodopsin, a photochemical cis-trans isomerization of retinal, and conclude in a transient change in the plasma membrane potential.¹⁸

The mechanism linking these two well studied processes has been the subject of many studies and speculations. Since this linking mechanism is the object of this study, some background experimental results and the currently accepted theory will be briefly presented.

One of the most striking aspects of vision is the small "amount" of light required. The threshold for visual excitation may be as low as one photon⁷ and only 1% bleaching is required for saturation response. This results in an amplification of about one million. Since only one rhodopsin is required to absorb a photon, each rhodopsin must somehow be linked to the output.

As previously mentioned, there appears to be no connection between the disc membrane containing rhodopsin and the plasma membrane. Thus, signals must be transmitted across the cell's cytoplasmic space. The distance from an activated rhodopsin to the plasma membrane can be as large as one micrometer.

Vertebrate rods containing large amounts of rhodopsin, typically 10^9 molecules.¹⁹ This indicates the excitation mechanism is capable of detecting much less than one part per million in altered receptors.

The result of transduction is a hyperpolarization (an increase in the negative potential across the plasma membrane) instead of depolarization. This is in contrast to the depolarization of nerve cells which is much better understood. A theory is required which somehow explains an increase in trans membrane potential.

The currently accepted theory of the ionic mechanism of transduction is due to Hagins and Yoshikami.¹⁹ They formulated their theory after having performed an extensive series of experiments dealing with the effect of various ions upon the plasma membrane potential. Originally, they had noted that when a rod is dark-adapted, current passively flows through the plasma membrane into the rod outer segment. The current is extruded somewhere along the inner segment. The inward flow results from the potential difference of approximately 50 millivolts (outside positive) across the plasma membrane. Researchers have not been able to further localize the sites of current flow. By varying the rod's bathing medium, Hagins and Yoshikami were able to show that Na^+ was required externally or the rod's response to light was abolished. However, upon allowing Na^+ to be replaced in the bathing medium, the response to light was completely renewed. Based upon this result, they concluded that the dark current is due to Na^+ flow, and the flow is somehow suppressed as the result of light absorption in the discs. Since the efflux of Na^+ continues in the inner segment, drawing Na^+ from the outer segment, the net result is an increase in membrane potential. (See Figure 1)

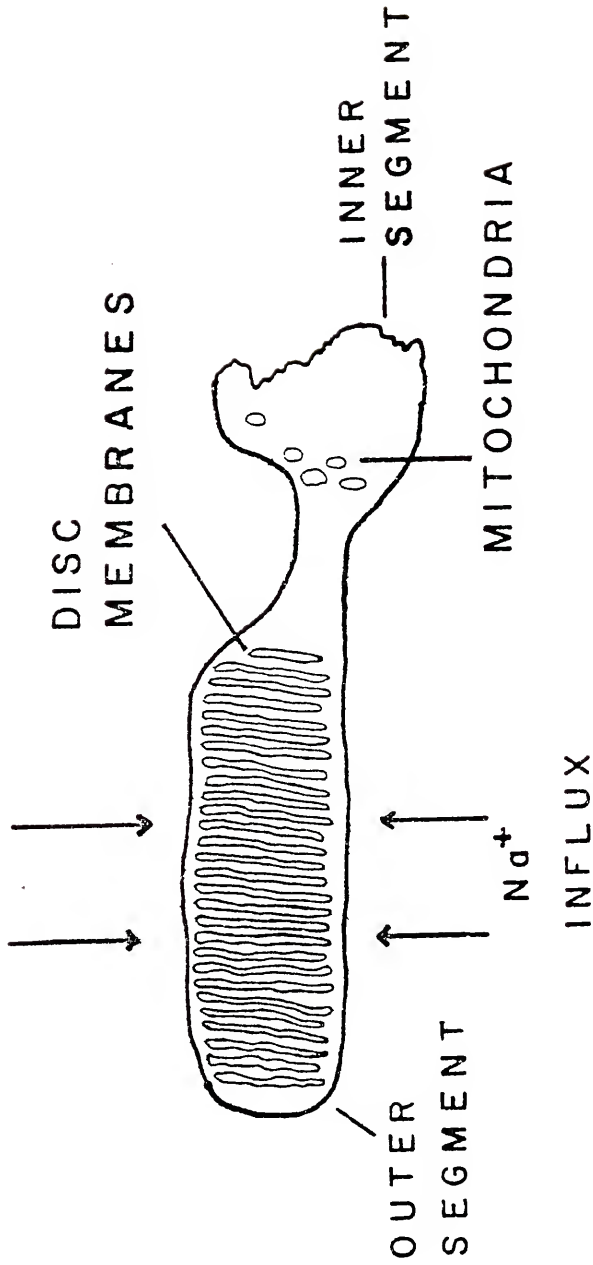


FIGURE 1
RETINAL ROD INDICATING DARK CURRENT

In an attempt to determine how the dark current into the outer segment is decreased, Hagins and Yoshikami injected various ions into the rod's cytoplasm. The result was that the addition of Ca^{++} had the same effect as light absorption, but the intracellular addition of K^+ , Cl^- , Na^+ , or Mg^{++} had no effect upon the membrane potential consistent with the absorption of light.

Due to the marked effects of Ca^{++} on both the dark current and trans membrane potential, Hagins and Yoshikami proposed that the dark current enters the outer segment through Na^+ specific channels located near possible Ca^{++} binding sites on the cytoplasmic (inner) side of the plasma membrane. If a Ca^{++} can prevent the influx of many Na^+ ions, the large amplification is easily explained.²⁰

To complete their theory, Hagins and Yoshikami proposed that Ca^{++} is initially sequestered inside the discs. The absorption of a photon by rhodopsin releases a number (3-300) of Ca^{++} ions from the discs into the rod's cytoplasm. Subsequently Ca^{++} diffuses to the plasma membrane, attaches at the appropriate sites, and reduces the Na^+ influx. Since the cytoplasmic side of the plasma membrane is already negative relative to the exterior, a reduction in Na^+ influx results in an increased trans membrane potential, a hyperpolarization.

CHAPTER IV

BACKGROUND EXPERIMENTAL RESULTS

Since Hagins and Yoshikami proposed their ionic theory of hyperpolarization, there have been numerous attempts to experimentally prove or disprove this theory. These experiments and others which indirectly yield evidence affecting their theory, will be discussed in this chapter.

Smith et al. examined the light-activated release of Ca^{++} from sonicated discs.²¹ They separated intact discs from bovine retinas, then placed the discs in a solution containing radioactive Ca^{++} . The solution was subjected to ultrasonic waves (sonicated), which tends to make the membranes leak. During this time, the radioactive Ca^{++} should have diffused into the discs. After sonication, the discs are stored allowing membrane resealing and retaining some of the radioactive Ca^{++} . The researchers then subjected the resealed discs to light while the effluent was being collected. Rhodopsin bleaching levels were between 24% and 57% and indicated a release of approximately 1 Ca^{++} per rhodopsin bleached. If the discs were not sonicated, they could not obtain any exchange with radioactive Ca^{++} . Therefore, they concluded that sonication is required to load discs with Ca^{++} . However, the authors were not able to show any Ca^{++} efflux with low light levels nor were they able to demonstrate that the discs were not disrupted by sonication.

Mason et al. performed a similar experiment.²² They obtained purified rod outer segments which were sonicated, incubated in a radioactive Ca^{++} solution, and resealed as vesicles. The authors claimed the resealed vesicles were similar to discs in appearance in electron micrographs. They were able to measure the ratio of Ca^{++} released to rhodopsin bleached with light levels between 5,000 photons per rod and 100% bleaching to be close to 1. In addition, they were able to show that sonicated discs actively accumulated Ca^{++} against a concentration gradient. This accumulation would be required in order to replenish the intradisc Ca^{++} supply.

The most extensive study utilizing radioactive tracers was performed by Sorbi and Cavaggioni.²³ They obtained frog rod outer segments which were passed through a microsyringe needle in order to burst the plasma membrane. The suspension was then placed in a medium containing radioactive ions.

They used radioactive K^+ , Na^+ , Rb^+ , Cl^- and Ca^{++} and were able to load the tracers without sonication. After loading, the discs were washed and subjected to varying amounts of luminous flux, ranging from a few photons per second per disc to almost saturation levels. Aliquots of the effluent were collected and analyzed for radioactivity. Low light levels did not alter the efflux of any of the radioactive ions. High light levels (approximately 50% bleaching) increased the efflux of Na^+ , Rb^+ , and Cl^- . The authors detected no change in Ca^{++} loss due to bleaching at any light level. Their experimental results placed an upper limit for Ca^{++} release due to light at a few hundred Ca^{++} ions released per disc. Thus, at high

bleaching levels they should have been able to detect the Ca^{++} changes predicted by Hagins and Yoshikami and a release of 1 Ca^{++} per rhodopsin bleached.

Liebman²⁴ used atomic absorption spectroscopy in an attempt to show a direct effect of light upon the Ca^{++} content of disc membranes. He obtained rod outer segments which were fragmented by vortexing in a medium containing Ca^{++} . This left only the disc compartment intact. Subsequently, the disc suspension was completely bleached or kept in the dark. He then determined Ca^{++} content by atomic absorption spectroscopy. His results indicated a Ca^{++} content which increased with increasing Ca^{++} concentration in the incubation medium and decreased with light exposure. For bovine rod outer segments in a 2 micromolar Ca^{++} solution, there were approximately 3.3 Ca^{++} per rhodopsin after bleaching and 4 Ca^{++} per rhodopsin before bleaching. However, the author felt that the time delay between bleaching and target preparation allowed re-entry of Ca^{++} into the discs. Subsequently he repeated the experiment with frog outer segments except that the medium contained a Ca^{++} chelating agent (an anion which strongly binds cations) which would prevent its re-entry. The results were that .2 Ca^{++} were released per rhodopsin bleached. It should be noted that these results were obtained with light levels much higher than physiological and yield a Ca^{++} release at least an order of magnitude lower than required by the theory.

An extensive study which was designed to use low light levels was completed by Szuts and Cone in 1977.²⁵ They used dark-adapted bullfrog retinas, measuring the Ca^{++} and Mg^{++}

contents of both discs and rod outer segments and attempting to detect changes due to physiological light levels. The light levels used ranged from approximately .001% to 1% bleaching. The authors designed their experiment to be done at low levels because rods function at low levels and are overridden by cone functions at higher light levels.

Retinas were shaken into a sucrose medium yielding outer segments which were exposed to the appropriate amount of light. (In some instances, Ca^{++} was added to the sucrose medium.) Immediately after exposure, a hypotonic solution containing a chelating anion was added to burst the outer segment plasma membrane and chelate any released Ca^{++} . The suspension was centrifuged to pellet the discs and the pellet prepared for atomic absorption spectroscopy measurements. Results indicated that neither disc nor rod Ca^{++} levels depended upon Ca^{++} levels in the external medium. This is in marked disagreement with Liebman's results.²⁴ Calcium content of discs was found to be approximately 1 Ca^{++} per rhodopsin. They were not able to detect any statistically significant change in Ca^{++} or Mg^{++} due to light absorption. They even found that in some samples Ca^{++} levels were higher in samples exposed to light than in samples which remained in the dark. The authors felt that they could have detected a 20% change in Ca^{++} content. Since this is much higher than that required to satisfy Hagins and Yoshikami's theory, they concluded that they were not able to definitively test the theory.

A similar experiment conducted by Hendriks et al.²⁶ was done at higher light levels and indicated Ca^{++} release upon

light exposure. These researchers used bleaching levels greater than 50%. They found that Ca^{++} content in dark-adapted frog rod outer segments was 11 Ca^{++} per rhodopsin if they were separated in a solution containing 3 millimolar adenosine triphosphate (ATP). The outer segments were either lysed and bleached or bleached and then lysed. In both cases, a 20% loss of Ca^{++} was reported. For their reported Ca^{++} levels, this is a 3 Ca^{++} released per rhodopsin bleached.

An interesting experiment done by Tam et al.²⁷ indicates Zn^{++} may be an important trace element in the functioning of visual excitation. Initially, the authors extracted rhodopsin utilizing the detergent emulphogene. The extract was exposed to light and then subjected to column chromatography which separates out the rhodopsin fraction. This fraction was analyzed for Mg^{++} , Ca^{++} and Zn^{++} content. The results were 1.4 Ca^{++} , 1.4 Mg^{++} and .8 Zn^{++} per rhodopsin in non-exposed samples. The levels of Ca^{++} and Mg^{++} were not dependent upon illumination; however levels of Zn^{++} in illuminated samples were 60% higher than in non-illuminated samples. Bleaching was 100% since the authors subjected the samples to light until no absorption at 498 nanometers was detected in the rhodopsin fraction. The value of .8 Zn^{++} per rhodopsin clearly is not stoichiometric, but the authors feel this is due to Zn^{++} losses in handling.

That rhodopsin is not a metalloprotein had been demonstrated many years prior by Fukami et al.²⁸ For their study, they used frozen retinas. Tam's group found that if they froze their preparation at any stage, Zn^{++} was completely lost.

These results appear to reopen the question of metallic ions bound to rhodopsin.

In addition to the previously described experiments attempting to directly measure Ca^{++} flux from photoreceptor membranes due to light exposure, there are numerous studies which indirectly indicate Ca^{++} may be involved in transduction.

Neufeld et al.²⁹ studied the binding of radioactive Ca^{++} to sonicated bovine rod outer segments. They found that Ca^{++} binding varied with incubation conditions; however, no light effect could be detected. The addition of ATP to the external medium more than doubled the amount of Ca^{++} bound. This result would be expected if disc membranes contain a Ca^{++} -ATPase for the active transport of Ca^{++} back into the discs.

Hemminki³⁰ utilized intact bovine rod outer segments which had been subjected to light or kept in the dark. Subsequently, all samples were placed in a medium containing radioactive Ca^{++} . He found that rods which had remained in the dark bound more Ca^{++} than rods which had been exposed to light.

Brown and Flaming³¹ repeated that portion of Hagins and Yoshimaki's experiment dealing with the effect of ions injected into functional rod outer segments. They studied the effect of injected Sr^{++} and Ba^{++} upon the rod outer segment plasma membrane potential. Sr^{++} had no effect; however, Ba^{++} reversed the effect of cytoplasmic Ca^{++} . An increase in intracellular Ba^{++} concentration increased the sensitivity of rods to light and more light was required to yield an equivalent hyperpolarization of the plasma membrane. The authors state that Ba^{++} has been detected in vertebrate retina and indicate

that it might have physiological significance. However, the presence of Ba^{++} in vertebrate photoreceptors has not been demonstrated.

One study which may contradict Hagins and Yoshikami was performed by Hess.³² She exposed frogs to light in vivo and compared Ca^{++} content of rod outer segments extracted from dark-adapted and light-adapted frogs. In addition, she exposed retinas extracted from in vivo dark-adapted frogs. She found that rods from in vivo light-adapted retinas contained 4 Ca^{++} per rhodopsin compared to .25 Ca^{++} per rhodopsin in rods from in vivo dark-adapted retinas. However, rods extracted from retinas which had been removed prior to light exposure and subsequently bleached contained only slightly more than .25 Ca^{++} per rhodopsin. These results indicate that upon light exposure in vivo, Ca^{++} is taken up by rods probably from the pigment epithelium. Since she used bleaching levels which were very high, it is not clear how (or if) this relates to the Ca^{++} hyperpolarization theory.

It appears that the presence of trace elements and their function in photoreceptor cells is unclear. This chapter contained two different methods of determining trace element content, radioactive tracers and atomic absorption spectroscopy. Most researchers using radioactive tracers have to sonicate their samples and then claim that upon resealing the vesicles are comparable to intact disc membranes. However, no one has presented any evidence that this is the case. The one study which did not sonicate their discs obtained vastly different results from those authors who did.

The other technique, atomic absorption spectroscopy, avoids the problem of sonication. However, the results obtained with this method do not agree any better than those obtained with radioactive materials. An explanation may be indicated in two statements by Szuts and Cone: "Even though our procedure was designed to minimize it, the unequal division of residual contamination by non-outer segment debris between test and control aliquots may have been the cause of the observed variability between experiments" and "in view of the variabilities we observed, it seems likely that an entirely different experimental approach will be needed to achieve such a low detection limit" (p 206).²⁵ However, the problem with atomic absorption spectroscopy is not one of detection limits, but is instead, one of not being able to detect small changes in a relatively high content.

CHAPTER V

X-RAY PRODUCTION BY PROTON BOMBARDMENT

The technique of particle induced X-ray emission (PIXE) depends on the high cross section for ionization and subsequent X-ray emission after excitation by a heavy, charged particle incident upon the target element. The various theories which interpret this process will be discussed in this chapter. (The next chapter describes the PIXE technique in detail.)

The simplest models assume the production of an inner shell electron vacancy is the result of the Coulombic interaction between the incident particle and the bound electron. Three theories have been formulated to explain this interaction: the plane wave Born approximation (PWBA), the impulse approximation (also called the binary encounter approximation or BEA), and the impact parameter method.³³ The first two are valid at high energies (incident particle energies much higher than the electron's binding energy) and are more useful for the understanding of PIXE. The impact parameter method was developed for adiabatic scattering (incident energy close to binding energy) and will not be considered in this work.

It can be shown that the validity criteria for both the Born approximation and the impulse approximation are similar for K shell ionization by protons and can be expressed as:

$$(V.1) \quad E_p/U_k > 24$$

Where U_k = electron's K shell ionization energy
and E_p = proton energy.³³

This will be considered the "high energy" region and will always be valid for the experiments performed in this research.

In first order perturbation theory, the cross section for a transition is proportional to the absolute square of the matrix element of the perturbing potential:

$$(V.2) \quad \frac{d\sigma}{d\Omega} \propto \left| \int \psi_n'^* (\vec{R}, \vec{r}) V \psi_n (\vec{R}, \vec{r}) d\vec{r} d\vec{R} \right|^2$$

where V = interaction potential

$\vec{R}$ = position vector of the incident particle

$\vec{r}$ = position vector of the target particle

ψ_n' = wave function of the final quantum mechanical state

ψ_n = wave function of the initial quantum mechanical state

and $\frac{d\sigma}{d\Omega}$ = differential cross section per solid angle.³⁴

Using the PWBA with a Coulombic interaction between the incident particle and electron, the differential cross section can be written:

$$(V.3) \quad \frac{d\sigma}{d\Omega} = \frac{M^2 v'}{4\pi^2 \hbar^4 v} \left| \int \frac{\psi_n'^* (\vec{r}) z e \exp \left[\frac{i}{\hbar} (\vec{p} - \vec{p}') \cdot \vec{R} \right]}{|\vec{R} - \vec{r}|} \psi_n (\vec{r}) d\vec{r} d\vec{R} \right|^2$$

where M = reduced mass of the atom particle system

e = electron charge

v = speed of relative motion before collision

v' = speed of relative motion after collision

$\bar{p}$ = momentum of relative motion before collision

$\bar{p}'$ = momentum of relative motion after collision

ψ_n' = final wave function of the electron

ψ_n = initial wave function of the electron

z = projectile charge

h - Planck's constant divided by 2π

$i = \sqrt{-1}$

and the remaining symbols are as previously defined. (In going from equation V.2 to equation V.3, a choice of "initial" and "final" states has been made. This is equivalent to making a choice of the Hamiltonian of the system."

Approximating the initial and final states by hydrogenic wave functions and neglecting all screening effects, the authors show that an approximate expression for K shell ionization is:

$$(V.4) \quad \sigma = \frac{2^{20} \pi a^2 \eta_k^4}{45 Z^4} a_0^2$$

$$\text{where } \eta_k = \frac{mE}{MZ^2(\text{Rydberg})}$$

σ = total ionization cross section

M = mass of incident particle

E = energy of incident particle

m = electron mass

a_0 = first Bohr radius of hydrogen

Z = atomic number of target atom

and the other symbols were previously defined.³⁴ Thus, η_k is a dimensionless number of depending upon the energy of the incident ion and the energy of the electron.

In this theory, the cross section varies as the fourth power of the incident ion energy. Thus, the loss of energy by the beam as it passes through the target material will result in a decrease in the cross section for K shell ionization. (As will be discussed in the next chapter, this should be considered before PIXE targets are prepared.)

Another important prediction of equation V.4 is a smooth variation of the cross section as the energy changes. It also indicates the cross section is proportional to the inverse twelfth power of the target atom's atomic number. Thus, the K shell ionization cross section by heavy, charged particles is comparable in magnitude for atoms of similar atomic number and will decrease rapidly with increasing atomic number.

The binary encounter approximation considers the target electron as a free particle. The role of the nucleus is solely to establish the momentum distribution of the target electron. The cross section for the interaction of the incident particle with a free electron is then summed over all momenta compatible with an energy exchange which is greater than the ionization energy of the inner shell electron. Finally, it is weighted by the momentum distribution of the bound electron states. The derivation is somewhat lengthy and complicated and the resulting equations are lengthy and broken up into various integration regions dependent upon theoretical parameters. Therefore, the derivation will only be outlined and some comments made concerning the results. The derivation is presented in the review article by Vriens³⁵ and thoroughly discussed by Garcia et al.³⁶

For a heavy, charged particle incident upon an electron, the cross section per unit momentum transfer can be written:

$$(V.5) \quad \sigma_p dp = \frac{8\pi z^2 e^4}{v^2 p^3} dp \quad (\text{the familiar Rutherford scattering formula})$$

where p = momentum transferred from incident particle
to target electron

dp = incremental momentum transferred

and the other symbols are consistent with previous usage.

An electron will be ejected if the amount of energy transferred is greater than the ionization energy. Therefore:

$$(V.6) \quad Q_k = \int_{u_k}^{\Delta E_{\max}} \sigma_p dE$$

where Q_k = ionization cross section for the K shell

$\Delta E_{\max}$ = maximum energy which can be transferred to
the target electron.

Equation V.6 is valid only for one electron speed. In order to obtain the proper result Q_k must be integrated over all allowed momentum values for the electron.³⁶ Thus:

$$(V.7) \quad \sigma_k(v_1) = N_k \int_0^{\infty} Q_k(v_1, v_2) F_k(v_2) dv_2$$

where $\sigma_k(v_1)$ = total ionization cross section for the
K shell

N_k = number of equivalent electrons having
binding energy U_k

v_2 = electron speed

$f_k(v_2)$ = speed distribution function of the electron.
 And $Q_k(v_1, v_2)$ = ionization cross section as before except
 the explicit dependence upon v_1 and v_2 is
 indicated.

The speed distribution function $f_k(v_2)$ can be determined "classically" from the micro-canonical ensemble using hydrogen energy values or quantum mechanically utilizing hydrogenic wave functions. In either case the result is:

$$(V.8) \quad f_k(v_2) = \frac{32}{\pi} v_0^5 \frac{v_2^2}{(v_2^2 + v_0^2)^4}$$

$$\text{where } v_0 = \left(\frac{2E_t}{m}\right)^{\frac{1}{2}}$$

and E_t = total energy of the hydrogenic electron.³⁶

Utilizing equations V.7 and V.8 Garcia et al. obtained the total K shell ionization cross section.³⁶ Even though the result is somewhat more lengthy than the PWBA result, it also is a smooth function of incident particle energy and decreases with increasing atomic number of the target atom.

Clearly the two approximations were found using different approaches and would not be expected to agree very well. They can be easily compared if the expressions are properly scaled so that $U_k^2 \sigma_k/Z$ is plotted versus Em/MU_k . This plot is shown in Figure 2. The curves plotted for the PWBA contain an effective charge to account for electronic screening of the nucleus and an effective potential to account for the reduction in binding energy of the K shell electron due to the outer electrons. These modifications are incorporated into a simple

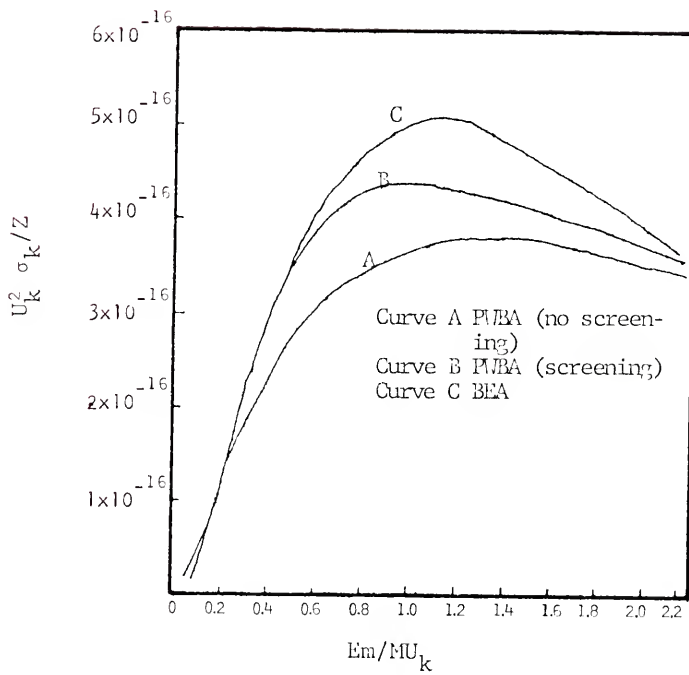


FIGURE 2

COMPARISON OF THE BINARY ENCOUNTER APPROXIMATION
AND PLANE WAVE BORN APPROXIMATION

parameter which is equal to one if all screening is ignored and decreases as screening is added. Curve A represents no screening and curve B a "reasonable" value of screening. The agreement between curve A and curve C (BEA) is reasonably good and if screening is added the agreement is improved significantly.

Many experiments have been performed measuring K shell ionization cross section and comparing the results with theoretical predictions. A summary of the experimental work done in the MeV range up to January 1976 is found in Table I of Johansson and Johansson.³⁷ Table I of this dissertation contains references to recent experiments which studied K shell ionization due to protons in the MeV energy range. The comparison between experiment and theory can be summarized by commenting that the theories are within 5% to 30% of experimental results in the energy range used in this study. This indicates that theoretical cross sections are probably not valid for absolute determinations, if accurate elemental analysis is desired.

Up to this point only the ionization cross section has been considered. However, once an inner shell vacancy is produced the ionized atom may rearrange itself by other mechanisms, the emission of an Auger electron or a Coster-Kronig transition. Coster-Kronig transitions do not occur for K shell vacancies³⁸ and will not be considered. Since Auger electrons are not detected in PIXE it is important to consider the relationship of the X-ray production cross section to the ionization cross section, which for a particular X-ray

TABLE I
RECENT K SHELL X-RAY PRODUCTION CROSS
SECTION STUDIES IN THE MeV RANGE

Author and Reference (a)	Year
Liebert et al. ³⁹	1973
Lear and Gray ⁴⁰	1973
Bearse et al. ⁴¹	1973
Gray et al. ⁴²	1973
Akselsson and Johansson ⁴³	1974
Criswell and Gray ⁴⁴	1974
Ishii et al. ⁴⁵	1974
Tawara et al. ⁴⁶	1974
Khelil and Gray ⁴⁷	1975
Twara et al. ⁴⁸	1976
Milazzo and Riccobono ⁴⁹	1976
Wilson et al. ⁵⁰	1977
Kover ⁵¹	1977
Tawara and Huchiya ⁵²	1977
Bissinger et al. ⁵³	1976

(a) All references are to reports of proton induced cross sections.

line is as follows:

$$(V.9) \quad \sigma_{xp} = \sigma_i w b$$

where σ_{xp} = X-ray production cross section

σ_i = ionization cross section

w = fluorescence yield (probability that the ion emits an X-ray)

and b = relative intensity of possible transitions to fill the inner shell vacancy.

The theory of fluorescence yields will not be discussed except to point out that all theories have predicted that the fluorescence yield is a constant for a particular K shell ionization within the projectile energy range utilized by PIXE.^{54,55} The importance of this will be clear after the next chapter. Table II gives fluorescence yields for ions of interest in PIXE spectra. It can be seen that they vary widely between light and heavy elements. The difference between elements studied in this work is approximately an order of magnitude. It is also obvious that the higher production cross sections for light elements are somewhat negated by lower fluorescence yields.

The other consideration in equation V.9 is the probability that the transition in question will occur to fill the vacancy. These probabilities have been extensively studied both theoretically and experimentally and are known to be constant for a particular inner shell vacancy.⁵⁵ (Two of the previous references, Bambynek et al.⁵⁴ and Salem et al.⁵⁵ are excellent

TABLE II
 FLUORESCENCE YIELDS FOR ELEMENTS USEFUL IN PIXE

ELEMENT	FLUORESCENCE YIELD (a)
P	.0604
S	.0761
Cl	.0942
Ar	.115
K	.138
Ca	.163
Sc	.190
Ti	.219
V	.250
Cr	.282
Mn	.314
Fe	.347
Co	.381
Ni	.414
Cu	.445
Zn	.479
Ga	.510
Ge	.540
As	.567
Se	.596
Br	.622
Kr	.646
Rb	.669
Sr	.691
Y	.711
Zr	.730
Nb	.748
Mo	.764

(a) All fluorescence yields are taken from reference 56.

review papers on fluorescence yields and transition probabilities respectively.)

An area which could be extensively discussed in this chapter is the energy dependence of the spectra obtained in X-ray emission studies. It is obviously important that the researcher be familiar with the energy levels and selection rules for atomic transitions.

Inner shells of atoms tend to strongly reflect the interaction of the nucleus with the inner shell electron. (This in part justifies the use of hydrogenic wave functions in describing inner shell processes.) As the nuclear charge increases, the nucleus binds the inner shell electron "tighter". The electrons also repulse each other, the outer shell electrons "pushing" the inner shell electrons "closer" to the nucleus. Both of these effects spread out the allowed energy levels as the atomic number increases. This results in higher energy transitions for heavier atoms. Thus, the energy of a particular transition varies systematically with the atomic number, and can be used as a means of detecting and distinguishing among the elements.

The X-ray spectrum must follow the quantum mechanical selection rules for electric dipole transitions. (Electric quadrupole and magnetic dipole transition probabilities are lower by a factor $(Z_{\text{eff}}/137)^2$.⁵⁷ Thus, electric dipole transitions will contribute the most intense lines for the elements in this study.) These selection rules eliminate most transitions and give rise to relatively simple X-ray spectra.

In spectroscopic notation, transitions to the lower ($n=1$) shell are referred to as K transitions. A transition to the next highest shell ($n=2$) is an L transition. (Transitions to other shells are lettered, but are not of interest in this paper.) This study predominantly involves K shell transitions. (One L line will be considered.) The theories of L shell vacancy production and fluorescence yields are much more complicated and not as well studied as those of K shells.

The K shell transitions are divided into two groups, K_α and K_β transitions. K_α transitions represent electrons falling from the $n=2$ (L shell) to the $n=1$ (K shell). K_β transitions include all other X-rays emitted when a higher shell electron fills a K shell vacancy. K_α and K_β energy levels for transitions in the commonly used PIXE energy range are shown in Table III. K_α and K_β transitions can be subdivided into transitions with different energies. This is not normally done in PIXE work because of limitations in detector resolution.

The X-ray lines which are commonly used in PIXE are shown in Figure 3. Electric dipole transitions are discussed in most graduate physics quantum mechanics texts and also the classic work of Condon and Shortley.⁵⁸ The only requirement of X-ray transition probabilities and energies is that they are consistent for a particular inner shell vacancy. This has been shown to be true for K shell vacancies of all atoms studied.⁵⁶

TABLE III
K SHELL X-RAY ENERGIES FOR ELEMENTS
USEFUL IN PIXE

ELEMENT	K_{α} (a)	K_{β}
P	2.015	2.142
S	2.308	2.468
Cl	2.622	2.817
Ar	2.957	3.191
K	3.313	3.589
Ca	3.691	4.012
Sc	4.090	4.459
Ti	4.510	4.931
V	4.952	5.427
Cr	5.414	5.947
Mn	5.898	6.492
Fe	6.403	7.059
Co	6.930	7.649
Ni	7.477	8.265
Cu	8.047	8.907
Zn	8.638	9.572
Ga	9.251	10.263
Ge	9.885	10.984
As	10.543	11.729
Se	11.221	12.501
Br	11.923	13.296
Kr	12.648	14.120
Rb	13.394	14.971
Sr	14.164	15.349
Y	14.957	16.754
Zr	15.774	17.637
Nb	16.614	18.647
Mo	17.478	19.633

(a) Energies are in keV

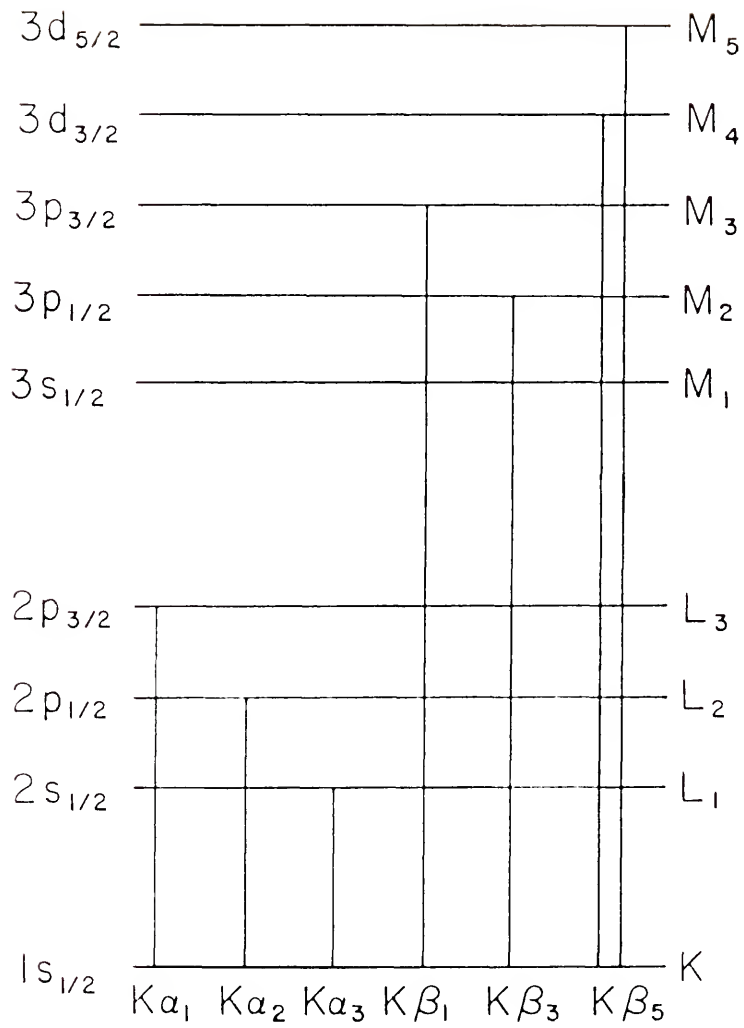


FIGURE 3

MAJOR K SHELL X-RAY TRANSITIONS

CHAPTER VI

PARTICLE INDUCED X-RAY EMISSION

In 1970, a study was published by Johansson et al.⁵⁹ demonstrating the feasibility of combining X-ray excitation by heavy, charged particles with an energy dispersive X-ray detector as a sensitive multi-elemental analysis technique. Since that beginning, the field has grown such that during a 1978 small accelerator conference in Denton, Texas, 19 papers were presented utilizing particle induced X-ray emission.⁶⁰ This chapter describes the PIXE technique and attempts to indicate why it is so useful. (The best review article available on the PIXE technique is by Johansson and Johansson³⁷ and is the source of most of the material in this chapter.)

PIXE requires a beam of charged particles, a target, and an X-ray detector. Typically the ion beam is obtained from a Van de Graaff accelerator; however, other types of accelerators have been successfully used. The target which consists of material to be analyzed is placed in the beam's path. One of the effects of the beam upon the target is to remove inner shell atomic electrons. The theories describing this process were discussed in the previous chapter. The ionized atom can rearrange by emitting characteristic X-rays which are detected in a suitable X-ray detector. As previously mentioned, the energy of the X-ray allows the detection of the element responsible for its emission.

Target preparation is an important part in any PIXE experiment. A researcher wants to alter the target as little as possible; however, the constraints imposed by the PIXE technique usually require some preparation.

The most obvious requirement for most PIXE targets is that they must be stable in a vacuum. (It is possible to perform PIXE with the target in air utilizing an external beam.) This means that biological samples must be subjected to some method of water removal. Ideally a drying procedure would be rapid, non-contaminating, simple, and remove only water from the target. Air drying, freeze-drying, vacuum substitution and ashing (both low and high temperature) are most often used. Air drying is the simplest choice, but the length of time required can be a disadvantage. However, it probably remains the most common method of water removal from biological samples. The other methods require appropriate equipment, are more involved, and can remove volatile elements from the sample.

The next requirement for target preparation is that both the absorption of emitted X-rays in the target and energy losses of the incident beam due to the target be considered. In the preceding chapter, it was shown that the cross section for ionization depends upon the incident particle energy. Since the beam particles lose energy during the interaction with outer shell electrons, the cross section would depend upon the target thickness. Fortunately, this can be ignored if target thickness is no greater than 1 milligram per square centimeter. This is often stated in papers reporting PIXE

experiments without reference to any calculations. It is shown to be the case in Appendix A. The absorption of X-rays, particularly softer X-rays emitted from elements which are of biological interest, is a different problem. Studies on X-ray absorption indicate that a target thickness of one milligram per square centimeter would require a 20% correction for light trace elements of biological interest (e.g., S, Ca and K). Many researchers attempt to make very thin targets and subsequently ignore X-ray absorption in the sample, while others only look at elements with high energy X-rays for which the absorption is not as great. However, for targets of typical PIXE thickness (milligrams per square centimeter) and elements in the range $Z < 20$ some type of correction must be made for accurate results.

After the water has been removed from the target material it must be formed into a thin target. For most materials, this requires the use of a backing for strength. Commonly used backings and the advantages and disadvantages of each are given in Table 6 of Johansson and Johansson.³⁷ If the target material is a serum or in solution, it may be placed directly upon the backing; however, this often leads to targets which are not uniform. If the data reduction depends upon target uniformity this can be a serious problem. Tissue samples probably present the most difficulty with target preparation and numerous methods have been employed to overcome these.³⁷ The final type of target is one in which a strong backing is used to filter the desired target materials from a fluid. This is done in aerosol samples and in water contamination studies.

Since the fluid passes through the filter, the target is immediately ready for analysis.

The above discussion should indicate some of the characteristics of a good backing material. It should be thin but strong, easy to handle, resistant to the target (some target materials destroy the backing), stable in an ion beam, and contain a minimum of contaminants. The diversity of target preparation methods and backing materials used attest to the fact that each problem must be considered individually.

Emitted X-rays can be detected using any type of X-ray detector. However, the use of a wavelength dispersive detector defeats the multi-elemental nature of PIXE or would require very long runs to obtain a complete spectrum. Therefore, almost all PIXE work is done with energy dispersive detectors and predominantly with lithium-drifted silicon Si(Li) detectors. These detectors have an energy resolution of approximately 150-180 electron volts (when new) with a 30 square millimeter detector area.

Since the ultimate sensitivity for PIXE will be strongly influenced by signals which do not result from the desired X-ray transitions, the sources of X-ray background in PIXE experiments will be briefly considered.

The background spectra can be divided into three parts. One part is due to bremsstrahlung from the incident particles. Most of the high energy background is due to this process. (For example, Figure 5 is the X-ray spectrum of a thin formvar film which contains no elements with X-ray transitions in the energy range shown. The X-rays above channel number 200 are

almost solely attributable to this source.) The largest portion of the low energy background is due to bremsstrahlung from secondary electrons. This results from the interaction of electrons removed from the target atoms with the matrix. Therefore, this portion of the background is due to the same process which produces characteristic X-rays, the production of electron vacancies.⁶¹

The third process contributing to the X-ray background is due to nuclear excitation of the target atoms by the incident beam, emitting γ -rays which result in Compton scattering in the detector. This process is hard to predict theoretically and is often ignored by researchers who use low energy beams. However, it can be modeled and is almost constant with X-ray energy in the energy range of Si(Li) detectors. It can also be decreased by an appropriate choice of beam energy and particles (i.e., protons and low energies).⁶²

It is clear from the previous discussion that the choice of beam energy can effect PIXE background and hence sensitivity. Also, as Figure 2 indicates, the cross section for inner shell ionization can be maximized by an appropriate choice of particle energy. Thus, the choice of a beam energy is a factor which should be given considerable thought in a PIXE experiment.

In addition to beam energy, sensitivity also depends upon the amount of charge incident upon the target. This can be seen considering the relationship between peak area and background under the peak. The criterion normally used for statistical significance is:

$$N_P \geq 3 \sqrt{N_B}$$

where N_P = number of X-ray counts in the peak

and N_B = number of X-ray counts in the background
under the same peak.³⁷

Therefore, an increase in total charge incident upon the target results in an increased ability to detect an X-ray peak.

Using this criterion and conservatively estimating parameters for detector resolution (165 electron volts), solid angle ($.003 \times 4\pi$), target thickness (.1 milligram per square centimeter), and total charge (10 microcoulombs), Johansson and Johansson predict minimum detectability curves for an "organic" target.³⁷ These curves are shown for two energies in Figure 4. They indicate that two MeV is a good choice of energy for biological work. (This also is a low enough energy to avoid γ -rays from the prevalent biological matrix elements, C and O.)

Another factor which must be taken into account in PIXE is the beam uniformity. If a nonuniform beam is incident upon a nonuniform target (nonuniform in directions perpendicular to the beam), erroneous data may be obtained. However, this can be avoided if either of the two is uniform. Most PIXE researchers produce uniform beams through the use of a beam sweep, diffuser foils, or beam defocusing.³⁷

One of the most difficult problems in obtaining quantitative data is calibration. As previously mentioned, theoretical cross sections are not suitable if accurate results

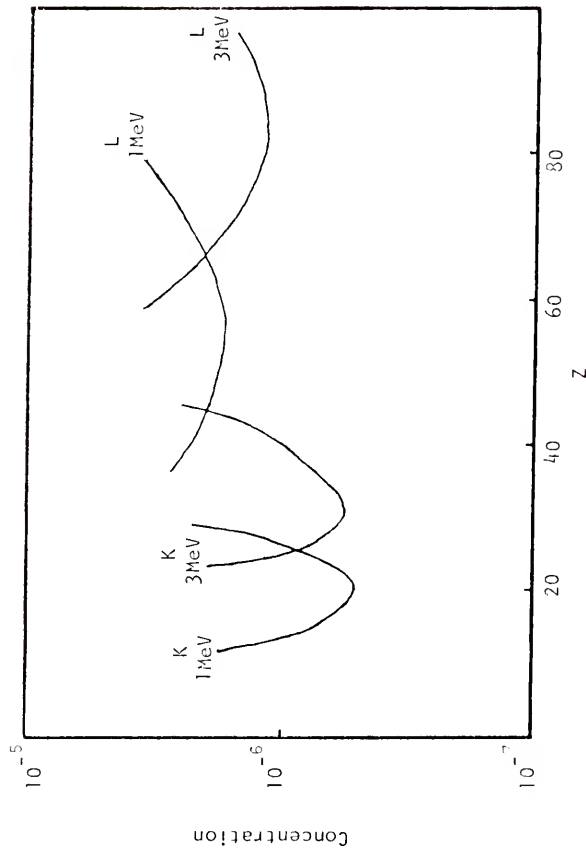


FIGURE 4
MINIMUM DETECTABILITY CURVES FOR PIXE

are wanted. Instead, most researchers have used one of two calibration methods. One is the incorporation of an "internal" standard. In this procedure a known amount of an element not present in the target material is added. The amount of one element in the target is now known and can be used to determine the amounts of other elements. The other method, which is more common, is the use of "external" standards. Standards for which the composition is known are subjected to a known amount of charge. The number of counts under each X-ray peak yields an absolute "system efficiency curve" which contains the probability of an X-ray being detected per atom per unit charge. The use of "internal" standards also incorporates a "system efficiency curve," but it is relative and relates the system efficiency of each element to the others. An interesting variation of a relative efficiency curve and external standards is used by Lear et al.⁶³ In this method a relative efficiency curve is determined utilizing external standards while the backscattered particles are monitored. The number of backscattered particles is then used to relate the target to standards.

The most difficult problem in PIXE experiments is spectrum analysis. Most samples of interest can contain many peaks upon a background due to effects previously mentioned. The most common procedure is to computer fit the X-ray peaks to Gaussian curves. This can be accomplished relatively easily unless interfering lines are encountered. The handling of overlapping peaks can present considerable difficulty and is handled in various ways by PIXE researchers.

There are numerous computer programs which are used to fit PIXE spectra. Most are modified nuclear physics programs and are not able to properly model PIXE background. Absorption by the target is considered by only one of the fitting programs. Absorption by the system components, windows to the X-ray detector, air gaps and external absorbers, are already included in the "system efficiency curves." Thus, if data are wanted on low Z elements in biological targets, the limitation will probably be in the fitting program.

There are many other aspects of PIXE which have not been mentioned in this chapter. Some are not pertinent to this study, others will be considered in subsequent chapters dealing with experimental procedures, and the remainder were not considered important enough for inclusion.

CHAPTER VII

PROCEDURE

The previous chapters have dealt with a diversity of subjects. Initially, the problem of transduction in photoreceptors was outlined, including the relevant experiments, and the dominant theory. Experimental attempts to verify the Ca^{++} transport theory were mentioned. The subsequent chapters tried to indicate that PIXE offered promise as a technique which could be utilized in this research, and included a brief theoretical foundation and description of the technique. (There seems to be no doubt that PIXE can have a tremendous impact upon clinical work; however, there have been few attempts to apply it to "fundamental" biophysical problems.)

The remaining chapters will describe the experiment which attempted to utilize PIXE in the determination of ionic content to disc membranes and the detection of changes due to light exposure, and the results of this experiment.

Bovine retinas were purchased from Hormel (Austin, Minnesota). The procedure used by Hormel to collect and freeze the retinas is described in Appendix B. The retinas, contained in vials of 50, were shipped in an insulated container and placed between layers of dry ice. The shipments were made by air freight. When each order was received, it contained

some dry ice. Therefore, it was felt that the retinas had remained frozen during the shipment. They were then stored in a freezer at -10°C .

When disc membranes were needed for an experiment, they were prepared using a separation procedure published by Smith et al.⁶⁴ This procedure is described in Appendix C. All preparation was done in a darkroom using dim red light filtered with a Kodak 1 filter. Prior to beginning the separation, the retinas were allowed to thaw in the dark for approximately two hours. Shorter thawing times reduced the yield significantly.

After separation, the intact disc membranes were suspended in five milliliters (ml) of 5% sucrose solution. The solution was divided into a number of equal parts (typically eight), placed in small transparent vials, and stored on ice until used.

For light exposure, the vials containing discs were placed on a counter at a predetermined location and subjected to a predetermined amount of light. For each vial subjected to light, a control was located at the same distance from the light source, but was covered with an opaque object. For the entire study there are equal numbers of control and exposed tubes. The levels of exposure ranged from approximately .1% bleaching to nearly 100% bleaching. For the calculation of bleaching levels, see Appendix D.

Immediately after being exposed to light (or used as a control), the vials were placed in a Beckman "Microfuge" and spun at 11,900 revolutions per minute for one or five minutes.

(The force during this time was 8874 xg.) This sedimented most of the disc membranes. After the supernatant was removed, the sediment was pipetted onto previously prepared thin formvar films. The top of the sediment was easily distinguishable from the sucrose medium and was completely removed. Therefore, the amount of sucrose medium remaining in the sediment should be proportional to the amount of sediment in the target. (This has been assumed by previous researchers attempting to measure ionic content. This was originally a necessary assumption for this work, however, as will be seen later, is not required.)

Before target preparation, but on the same day, 2% formvar backings were made. These were discarded if not used that day. The choice of backing has been considered previously. Formvar was chosen for this work because it can be made with a lower level of contaminants than other easily produced backings. The fact that formvar can be made very thin was also a factor in its being used. The films were floated on double distilled water and picked up on aluminum frames.

After the targets were prepared, they were placed in a closed container to air dry. This required two or three days depending upon the amount of material in the target. Blank formvar backings remained with the targets at all times after they were prepared. This was to ensure there were no contaminants due to external contamination.

Targets were then taken to Florida State University (FSU), Tallahassee, Florida, where they were subjected to PIXE analysis. The proton beam of five MeV was obtained from the FSU

FN accelerator. (This is clearly too high an energy for optimizing sensitivity for the elements in this study. However, the most important limitation upon beam energy is what can be obtained and the FSU FN accelerator does not run well at lower energies.) The beam was made uniform by means of a beam sweep and subsequently collimated by the use of a carbon collimator of diameter 4.5 millimeters located one meter in front of the target chamber. The accumulated charge and beam current were monitored by a Faraday cup located past the target. Beam currents were kept low, approximately five nanoamperes, in order to prevent pulse pile-ups in the X-ray detector. Each target was analyzed twice, once for an accumulated charge of one microcoulomb and then for an accumulated charge of 20 microcoulombs. (The reason for this will be explained later in this chapter.)

The resulting X-rays were detected in a Si(Li) Kevex detector with a measured full width at half maximum of 200 eV at 6.4 keV. Count rates were between three and five thousand counts per second. The exit window from the PIXE chamber was .0064 mm mylar. The total air gap between the exit window and detector window was seven millimeters. And the detector window was .013 mm Be. The vacuum in the PIXE chamber was below 10^{-5} torr; therefore, no correction for X-ray absorption in the chamber was made. The target was located in a vertical plane at an angle of 30° from the beam. The X-ray detector was located 90° from the plane of the target.

The PIXE spectra were then fit with the computer code HEX which was developed especially for PIXE spectra at FSU

by Henry Kaufmann.⁶² HEX is one of the computer codes which is able to account for target absorption of X-rays. It is described in Appendix E.

In studies of disc membranes, it is preferable to relate ionic composition to the amount of rhodopsin. This enables ionic changes to be easily compared to the amount of rhodopsin bleached. Normally this is accomplished by taking equal aliquots from the same suspension and assuming they contain equal amounts of both contaminating material and rhodopsin. When this work was begun, this procedure was attempted; however, it was not possible to equalize rhodopsin in this manner. This could easily be seen by looking at the "equal" aliquots. There are methods used which tend to better yield "equal" aliquots; however, it was felt that a better method could be devised through the use of the PIXE technique.

A second problem relating to the amount of material present is the requirement for an accurate determination of the amount of sample. This is normally done by weighing the dried sample. However, the reproducibility of air drying may not be good enough to use this as a base for determining ionic changes.

With both of the preceding problems in mind, plus a desire to lower the PIXE calibration error as much as possible, an attempt was made to find an element which could be used as a true internal standard. It would be preferable if this element were contained in rhodopsin, were not found in the rest of the disc membrane nor the cytoplasmic space of the rod outer segment and were not present in any chemicals used.

Fortuitously there seems to be such an element, sulfur. It is well known that rhodopsin contains sulfurs as both disulfides and sulphydryls. The reactions of sulphydryl groups in rhodopsin have been studied by various research groups.^{65,66} The number of sulfurs in rhodopsin is still debated with ten and twelve being the most accepted numbers. Chen and Hubbell's number of ten sulfurs per rhodopsin will be used in order to calculate ionic composition per rhodopsin.⁶⁵ However, the number of sulfurs does not matter if only changes in ionic composition are wanted.

It cannot be unequivocally stated that sulfur is not contained in disc membranes in other than rhodopsin; however, the lipid components are known and do not contain sulfur. The remaining protein component in disc membranes is not known. Rhodopsin is 85% of the membrane protein and it could be assumed that most of the membrane sulfur would be in rhodopsin. If there were other sulfurs in the disc membrane, the amount of sulfur would then be a measure of the disc membranes in the target material. And as before, the changes in ionic composition would not be effected. Regardless, for the remainder of this study, it is assumed that all disc membrane sulfur is in rhodopsin and that each rhodopsin contains ten sulfurs.

Because the X-ray production cross section for light elements is so large and they are usually present in high concentrations, the X-rays from these elements dominate the X-ray detector in the configuration described earlier. For better detection of heavier elements, an external absorber is placed into the X-ray path. This absorbs most of the low energy

X-rays, but transmits most of the higher energy X-rays. For each PIXE target, one run was made without an absorber yielding predominately X-rays from light elements, and then a run was made with the external absorber giving a larger contribution from heavier elements.

A source of possible contamination by other than membrane sulfur is sulfur contained in free amino acids. Taurine is a commonly occurring sulfur containing amino acid which is found in retinas. However, it has been shown to be absent in rod outer segments.⁶⁷

Another type of contamination which could affect the results is due to other cell organelles. Since the outer segment does not contain organelles other than disc membranes, if the preparation were pure, this would not be a problem. However, mitochondria are present in the inner segment and could be present in the suspension after the outer segment is broken off from the retina. Mitochondria contain large amounts of Ca^{++} and Cu^{++} which could lead to erroneous values for these elements.

An assay was performed to determine the presence of mitochondria. The crude rod outer segment preparation which was obtained after the first flotation on 45% sucrose was assayed for succinate dehydrogenase. Succinate dehydrogenase is an enzyme which is present in mitochondria and is commonly used as a mitochondrial assay. The amount of crude rod outer segment used in the assay was adjusted until activity was easily determined. Then a comparable amount of final disc membrane preparation was assayed. In this preparation, activity

was lower by a factor of 20. Therefore, it was concluded that mitochondrial contamination was not significant. The assay procedure is described in Appendix F.

CHAPTER VIII

EXPERIMENTAL RESULTS

Figure 5 is the X-ray spectrum of one of the formvar controls stored with the PIXE targets. The contribution due to bremsstrahlung, both incident particle and scattered electron, is clearly evident. There are no X-ray peaks present due to elements having energy transitions in this range. This is a typical formvar spectrum and indicates there was no contamination during air drying and storage. The amount of contamination on any formvar blank did not exceed .1% of the target amount.

Figures 6, 7, and 8 show the X-ray spectra of chemicals used in the separation procedure. Ficoll is seen to contain small amounts of Ca, approximately 100 parts per million. Sucrose contains Cu at an approximate level of two parts per million. The KPO_4 buffer is apparently free from any significant contamination.

Figure 9 is the X-ray spectrum of a disc membrane preparation. It was run without an external absorber and clearly shows the light elements P and S. Figure 10 is the same target run for a longer time with an external absorber. A comparison of Figures 9 and 10 indicates how the use of an external absorber "brings out" the heavier elements with respect to the lighter elements.

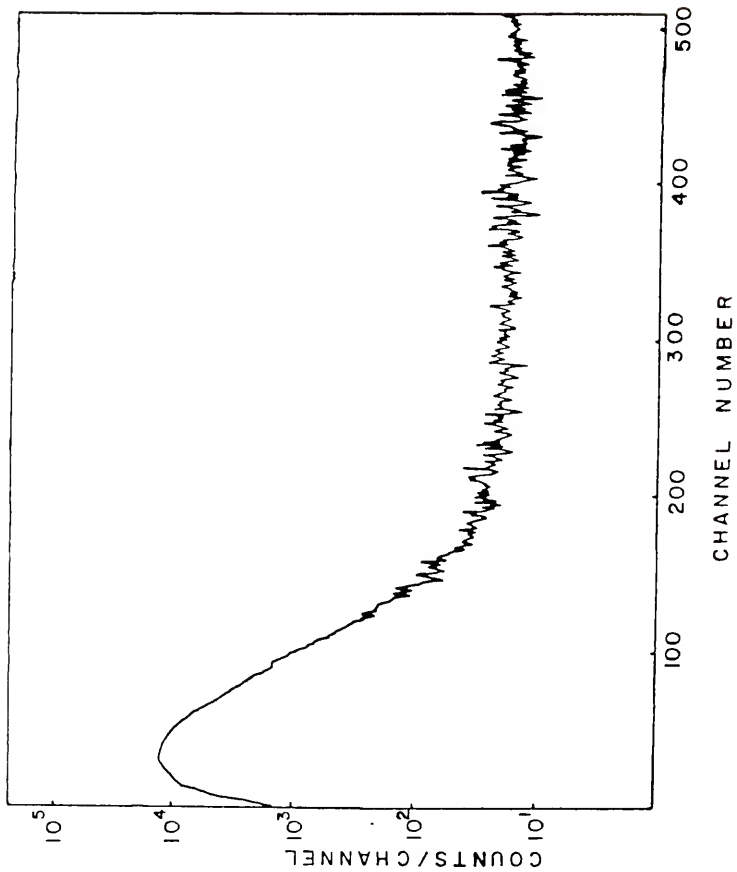


FIGURE 5
X-RAY SPECTRUM OF FORMVAR BLANK

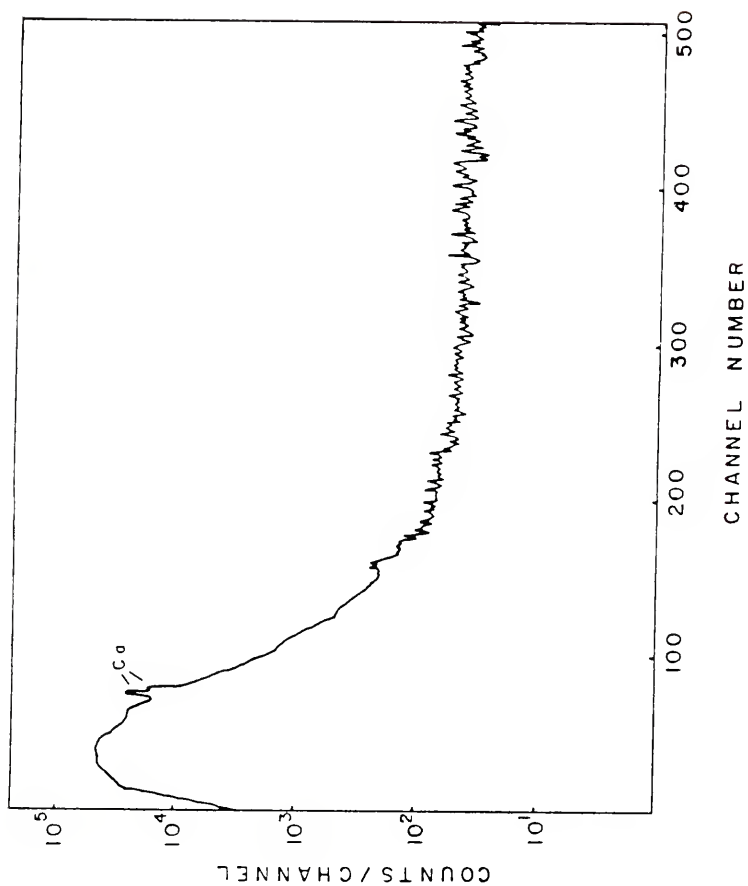


FIGURE 6
X-RAY SPECTRUM OF FICOLL

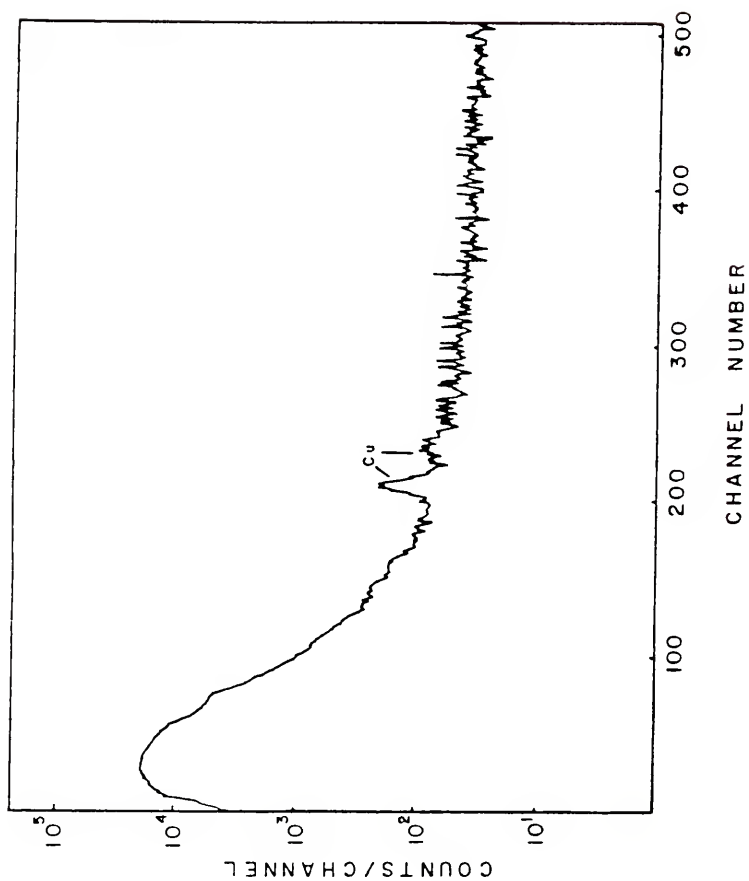


FIGURE 7
X-RAY SPECTRUM OF SUCROSE

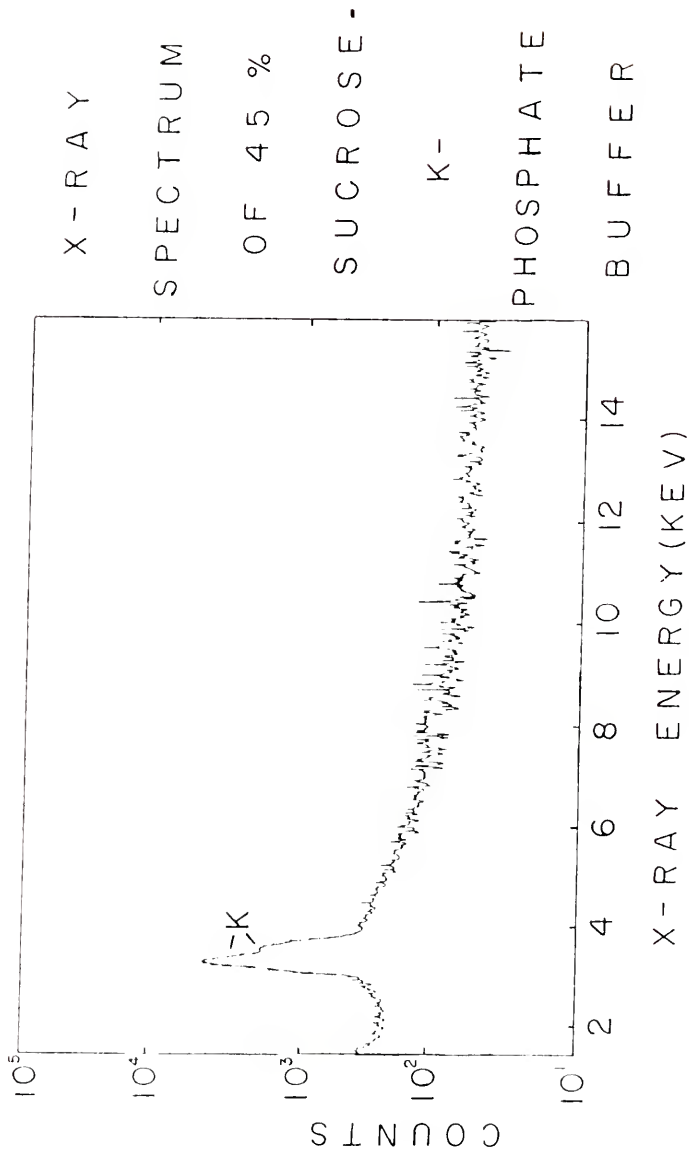


FIGURE 8

X-RAY SPECTRUM OF POTASSIUM PHOSPHATE BUFFER

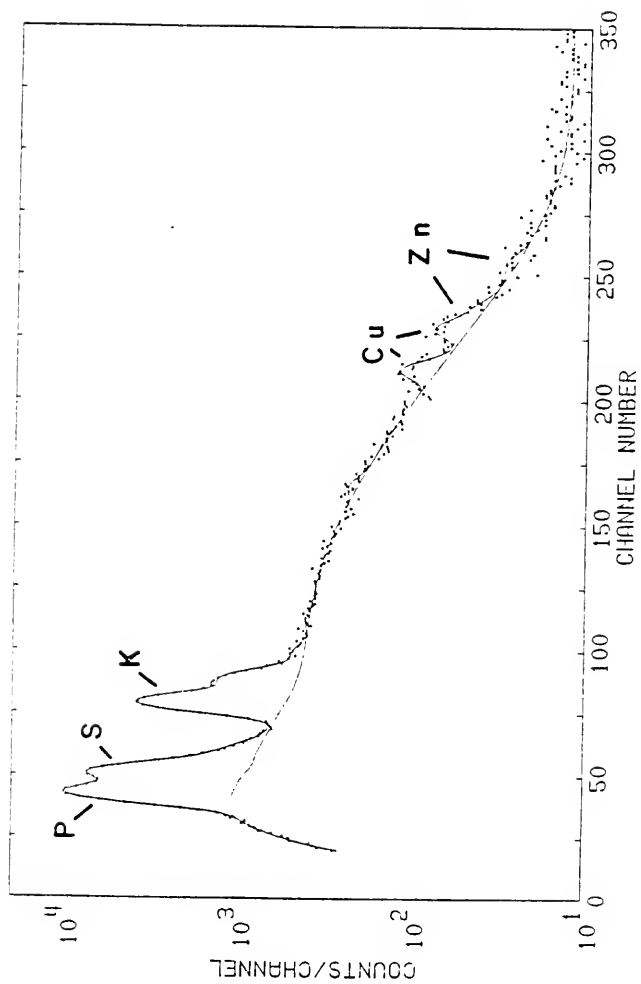


FIGURE 9
X-RAY SPECTRUM OF DISC MEMBRANES WITHOUT EXTERNAL ABSORBER

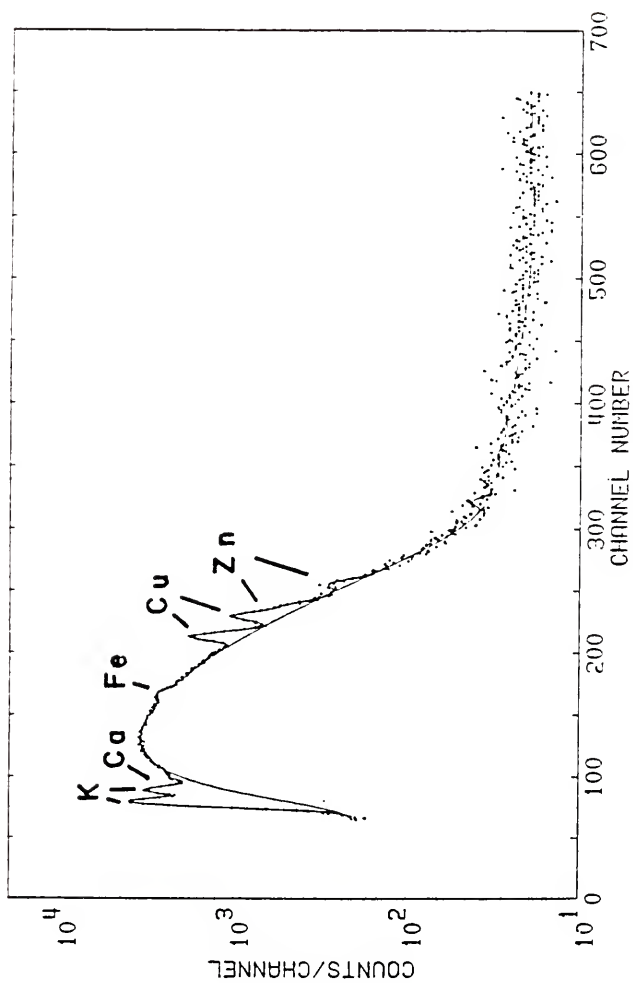


FIGURE 10
X-RAY SPECTRUM OF DISC MEMBRANES WITH EXTERNAL ABSORBER

The results for each target are given in Table IV. The value given for each element is with respect to rhodopsin making the previously mentioned assumptions on the S content of rhodopsin. Included is the tube from which each target was extracted and the amount of bleaching. No values are given for P or K since they were contained in large amounts in the chemicals used in the separation procedure. Therefore, these values would be unreliable as the composition of disc membranes. The range given for each value represents the error calculated from HEX combined with an error which represents the choice of matrix composition. Both of these are discussed in Appendix E.

Table V contains the amounts of the most prominent trace elements detected in disc membrane preparations. These values are determined by averaging over all of the control values. The range given is the standard deviation of the data.

Even though the major reason for performing these experiments was to determine the trace element composition of disc membranes, the presence of other elements can be detected as well as the absence of numerous commonly occurring biological trace metals. In addition to the trace elements listed in Tables IV and V, the amount of chlorine in each sample was determined. The mean value and standard deviation for all 22 samples are $.903 \pm .455$.

Since HEX looks for the presence of any element requested and is able to quantify the minimum concentration required for detection, it is possible to place an upper limit on the amounts of elements not detected. This was done for manganese, molybdenum and nickel and resulted in upper limits per rhodopsin of .007, .006, and .0033 respectively.

TABLE IV
TRACE ELEMENT COMPOSITION OF DISC MEMBRANE TARGETS

Target	Tube	Bleaching (a)	Ca(b)	Fe	Cu	Zn
1	A	1%	.571 ± .059	.0266 ± .0031	.257 ± .025	.0692 ± .0070
2	A	C	.435 ± .057	.0171 ± .0030	.208 ± .021	.0606 ± .0062
3	A	1%	.461 ± .052	.0228 ± .0037	.227 ± .023	.0672 ± .0071
4	A	C	.616 ± .066	.0286 ± .0043	.263 ± .026	.0754 ± .0080
5	B	1%	.519 ± .056	.0260 ± .0036	.134 ± .013	.0676 ± .0070
6	B	C	.529 ± .076	.0249 ± .0034	.131 ± .013	.1645 ± .0066
7	B	1%	.463 ± .049	.0201 ± .0025	.105 ± .011	.0532 ± .0055
13	A	100%	.451 ± .051	.0178 ± .0025	.217 ± .022	.0662 ± .0068
10	A	C	.303 ± .040	.0156 ± .0020	.133 ± .013	.0399 ± .0040
11	B	100%	.436 ± .047	.0177 ± .0024	.108 ± .011	.0532 ± .0055
12	B	C	.848 ± .088	.0142 ± .0026	.106 ± .011	.0532 ± .0056
21	C	.1%	.510 ± .074	.0253 ± .0026	.0930 ± .011	.0497 ± .0053
22	C	C	.412 ± .056	.0212 ± .0022	.0740 ± .0078	.0496 ± .0054
23	C	.1%	.512 ± .065	.0230 ± .0023	.0752 ± .0078	.0475 ± .0051
24	C	C	.458 ± .065	.0221 ± .0023	.0722 ± .0074	.0509 ± .0060
25	C	1%	.569 ± .064	.0472 ± .0049	.0923 ± .010	.0463 ± .0050
26	C	C	.529 ± .097	.0306 ± .0032	.106 ± .012	.0535 ± .0074
29	D	10%	.480 ± .080	.0348 ± .0037	.130 ± .015	.0630 ± .0082
30	D	C	.409 ± .063	.0339 ± .0035	.123 ± .014	.0627 ± .0031
31	D	100%	.395 ± .046	.0313 ± .0040	.0971 ± .010	.0539 ± .0062
32	D	C	.373 ± .046	.0346 ± .0049	.0989 ± .010	.0667 ± .0070
33	D	1%	.573 ± .066	.0437 ± .0055	.017 ± .012	.0663 ± .0071

(a) Bleaching levels are explained in Appendix D.

(b) All values are elemental content per rhodopsin.

TABLE V
TRACE ELEMENT CONTENT OF ROD OUTER SEGMENT
DISC MEMBRANES

Ca (a)	.492 ± .153
Fe	.0243 ± .0075
Cu	.132 ± .060
Zn	.0577 ± .010

(a) Calculated by using control values from Table IV.

CHAPTER IX

DISCUSSION, CONCLUSIONS, AND AREAS FOR FURTHER STUDY

The primary goals of this study were to determine the concentration of trace elements in dark-adapted frozen bovine retinal disc membranes and attempt to measure any changes due to light absorption. A secondary aim was to demonstrate the usefulness of the PIXE technique in fundamental biophysical research. This chapter discusses each of these, including comments upon possible metallo-enzymes which may be present in disc membranes. In addition, ideas for future research which continue the visual transduction problem and enter new areas in which PIXE offers unique capabilities are mentioned.

Table V shows the elemental content of disc membrane targets as determined by this study. All of the elements have less than a one to one stoichiometry with rhodopsin. However, this does not preclude higher levels in vivo with some subsequent loss during preparation.

The amount of calcium present in disc membranes agrees well with reported values by other researchers. Szuts and Cone concluded that the calcium content is 0.2 calcium per rhodopsin within a factor of two,²⁵ Hendriks et al. reported 11 calcium per rhodopsin (using a separation medium containing ATP),²⁶ and Hess reported 0.25 calcium per rhodopsin.³² These values all refer to dark-adapted disc membranes.

Even though none of the elements are stoichiometric with rhodopsin, the concentrations detected are high enough to indicate a biological significance. The amount of calcium is large enough to indicate it could be the transmitter in Hagins and Yoshikami's theory. Another role has been suggested for calcium by Hess.⁶⁸ Since she determined that calcium content in rod outer segments was greater after light-adaptation in vivo, she feels that calcium may have a role in light-dark adaptation. (As background light intensity is increased, the threshold for visual excitation also increases.) However, she did not describe a mechanism for a calcium dependent adaptation. Another possible role for calcium would be to modulate enzyme activity. It has been well established that calcium concentration is an important factor in several enzymatic rates.⁶⁹ Therefore, the calcium level might be controlled solely as a means of regulating enzymatic activity. It is also possible that such an enzyme mediated by calcium could be involved in visual transduction or light-dark adaptation.

The trace element with the next highest concentration in discs is copper. Hess had previously detected copper in intact rod outer segments and has suggested it may be due to the presence of superoxide dismutase.⁶⁸ Superoxide dismutase is a cytoplasmic enzyme containing two zinc and two copper ions per molecule and is responsible for destroying superoxide radicals. It probably doesn't contribute significantly to the values in Table V since the copper values vary widely. If this enzyme were present in large amounts, the zinc values would vary with the copper values. In addition, the enzyme is unlikely

to be present in the discs and any cytoplasmic enzyme should be lost during the washes after the rod outer segments are bursted. A role for copper in the functioning of disc membranes has not been suggested.

It may be recalled that the sucrose used contained copper as a contaminant. A solution of 5% sucrose containing 100 parts per million of copper would be .08 millimolar (mM) copper. Therefore a small part of the copper found may be due to contamination. However, the reason for the remaining copper detected is not presently understood.

The case for a membrane associated zinc binding protein appears to be much better. As Table IV shows, zinc values are relatively consistent, except for sample 6, and would indicate that zinc is closely associated with the disc membrane. A possible choice for this enzyme is carbonic anhydrase which has a molecular weight of about 30,000 and contains one zinc per molecule.⁶⁹ If all the zinc present were due to carbonic anhydrase, it would be present at a level of .14 mM (using a rhodopsin concentration of 2.5 mM)²⁴ and constitute approximately 4% of the membrane protein by weight. Carbonic anhydrase seems to be a good possibility because it maintains intracellular pH levels.⁶⁹ In a semi-closed system such as discs or rod outer segments, a mechanism would be required to maintain the internal pH. High fluxes of ions occur and the pH changes must be constantly compensated. If this were not the case, the pH could vary detrimentally from physiological levels.

As previously mentioned, Tam et al.²⁷ concluded there could be a one to one stoichiometry between zinc and rhodopsin in vivo, but they were unable to detect zinc in preparations from bovine retinas which had previously been frozen. Comparison of their results with those of this study is difficult. However, the separation procedure used here is "gentler" and might be expected to result in less zinc removal.

The concentration of iron is the lowest of the trace elements listed in Table V. Iron in biological systems is always bound to high molecular weight substances. It may be present in either a soluble protein or a membrane bound protein. There are many known iron containing membrane bound proteins.⁶⁹ Many of these function as electron carriers and some of these are present in mitochondria. Since the mitochondria assay showed highly reduced activity in the final preparation, it is felt that mitochondrial proteins are not the major contributor to the iron content of disc membranes. Due to the fact that iron containing proteins are widespread, its presence is not surprising and agrees with the results of Hess.⁶³ However, it is not yet appropriate to suggest a likely reason for its presence in disc membranes.

The amount of chlorine (which would be present as the chloride anion) is interesting. As the standard deviation of the data indicates, the chlorine content varies widely between samples. This is expected, as it probably is not closely associated with disc membranes. Instead, it would be present in the rod outer segment cytoplasmic space. Chloride could have a role in visual transduction, but this has not been postulated.

Since the possibility of a role for barium in visual transduction has been reported,³¹ an attempt was made to place an upper limit on the amount of barium present in disc membranes. The details of this estimation are contained in Appendix G and yield an upper limit of .02 barium atoms per rhodopsin.

The absence of manganese, molybdenum, and nickel above minimum detectability levels is significant because it shows there are no proteins which contain these trace metals at high concentrations in disc membranes. The upper limits determined are still high enough to allow the presence of minute amounts of a protein containing one of these metals as a part of the 15% non-rhodopsin disc membrane protein.

If the samples made from each tube are considered separately and the amounts of each element averaged, in almost every case the values are correlated better than those for all the controls. This is true even if the data for those samples exposed to light are included in the average for each tube. Therefore, there is a significant difference among the disc membrane solution in each tube. Since this experiment used tissues from animals whose diets are not controlled, this result should probably be expected. (In retrospect, one of the two major weaknesses of this study was the lack of any control over the animals including their diet, light exposure, slaughter conditions and retina removal.) Since 50, or 100, retinas are contained in a tube, it would be expected that the targets coming from the same preparation would be more homogeneous. The data indicate this is true.

Since it was felt that the values should be reproducible, at least in the control targets made from each tube, the conclusion was that the handling after the solution was divided could be contributing to the difference. The most obvious step to consider was the final sedimentation in the Microfuge spin which could have caused unequal losses in trace elements. Therefore, the spin time was lowered from five minutes for tubes A and B to one minute for tubes C and D. This resulted in zinc values which were relatively consistent in the samples made from tubes C and D and some improvement in the calcium, copper, and iron values.

The fact that the zinc values were made reproducible was taken as an indication that the method of calibration with respect to sulfur was valid and that only an improved procedure of handling the samples between light exposure and target preparation was required. Unfortunately, the scope of this study precluded an analysis of effects of various centrifugations and external medium upon the presence of trace elements. Recently some research has been performed in this area⁷⁰ and indicates that a single low speed centrifugation can cause up to a 60% loss of calcium. Any significant loss of trace elements during centrifugation is probably not very reproducible.

Previous attempts at detecting a light effect on calcium efflux have all relied on a similar type of centrifugation. However, in none of the reports of these studies is there an indication that unequal loss of trace elements during centrifugation was considered. In the experiment performed by Szuts and Cone, the pellet was "formed" approximately five

minutes after having been subjected to light.²⁵ The authors do not indicate that they considered this step to be a potential problem, but as previously stated ascribe the disparity to an unequal division of contaminating material. That possibility was considered in this study; however, the consistent ratio of zinc to sulfur seems to indicate that this is not the case. Since the final centrifugation is performed with phosphate buffer as the medium, it is felt that the differences in concentrations of calcium, copper, and iron represent unequal losses and not contamination from the medium. (This was the other major problem encountered during this study.)

The other major goal of this study was to detect changes in the previously mentioned ionic concentrations of disc membranes due to light absorption. The amounts of calcium, copper, and iron were not considered reproducible enough to detect changes due to low bleaching levels. However, this experiment could have detected calcium changes as reported by Smith et al.,²¹ Mason et al.,²² and Hendriks et al.²⁶ and zinc changes as reported by Tam's group. Each of these studies utilized high bleaching levels and the first three indicated at least a one to one release of calcium per rhodopsin bleached. These changes could have easily been seen in the targets which were totally bleached; 11, 13, and 31. A possible reason for not observing any changes in zinc has been mentioned. However, it must be concluded that changes in calcium due to high bleaching levels were not present under the conditions of this experiment. This agrees well with later more controlled attempts to measure calcium changes in disc membranes due to light absorp-

tion.^{23,25} This does not conclusively prove the calcium transduction theory wrong. It has been previously mentioned that the bleaching levels of bovine retinas obtained from Hormel are not controlled and could be high enough so that rods have been desensitized. That this is probably the case is indicated by Smith et al. who state that approximately 25% of the rhodopsin is bleached.⁶⁴ If true, it is possible that the calcium transduction theory of Hagins and Yoshikami can not be tested utilizing these slaughterhouse retinas.

One very significant result of this study has been that it has demonstrated the advantage of using PIXE as a tool for studying biological problems. This has also resulted in numerous ideas for future research, some of which will be briefly discussed.

A very important problem which should be studied is the determination of the ionic composition of dark-adapted disc membranes. For this purpose, animals (probably frogs or rats) should be dark-adapted in vivo, the retinas removed in the dark, and quick frozen. Then thin slices can be prepared with an ultramicrotome, placed on formvar backings, and examined with PIXE. In order to determine whether the slice has been made through rod outer segments, each slice can be examined under a light microscope. These targets can be made so thin that they can be subjected to Rutherford backscattering analysis to determine the low Z composition not obtainable with PIXE. This can be done at the same time characteristic X-rays are being detected for PIXE analysis. Since the matrix responsible for X-ray self absorption is made up of the low Z elements,

the matrix composition can be included in the computer code yielding much more accurate values for self absorption. In addition, the combined use of PIXE and Rutherford backscattering analysis allows the entire elemental composition, except for hydrogen, to be found.

As a modification of the previously mentioned study, the animals should be light-adapted in vivo and disc membrane targets prepared in the same manner. A comparison of the two experiments would not test Hagins and Yoshikami's theory, but it would clearly show if there are significant effects (which originate outside the retinal rod) upon the ionic content of disc membranes. This is of interest since the data of Hess³² indicate that calcium may be released into rod outer segments from the pigment epithelium during light-adaption. Each of these can be done by comparing the sulfur content to that of the element of interest as was done in this study.

Another study which could be easily done is to utilize PIXE as a verification of the number of sulfurs per rhodopsin. Disc membranes could be prepared by a suitable preparation technique and analyzed with PIXE. Using the percentage of rhodopsin in disc membranes and assuming that all the sulfurs in disc membranes are contained in rhodopsin will yield the number of sulfurs per rhodopsin.

As a result of this research, it is obvious to this author that any reasonable attempt to measure calcium changes in disc membranes due to light absorption must begin with animals which are known to be dark-adapted, must use a procedure for obtaining disc membranes which does not cause the loss of ionic

components, and should use a method of internal calibration similar to the one developed in this study utilizing sulfur. Since the theory of Hagins and Yoshikami predicts at least ten calcium ions released per rhodopsin bleached, it is felt that this experiment could definitively test the theory at low bleaching levels (1%) if losses during preparation can be prevented.

One promising clinically oriented study is to examine the effect of zinc deficient diets upon both retinal degeneration and retinal zinc content to include disc membrane and pigment epithelium content. This is needed research as there are indications that zinc deficient diets are related to the most common retinal disorder, retinitis pigmentosa. (William Dawson, Department of Ophthalmology, University of Florida, Gainesville, private communication). Rats, whose diets are easily controlled, could be used in this study with existing physiological techniques used to monitor the extent of retinal disorder and PIXE used to determine the corresponding trace element content.

The previously suggested research is all related to the work done in this study. However, it should be obvious that the PIXE technique can be applied throughout many biological research areas and seems to be uniquely suited for research in some of these. One broad area suitable for PIXE is that of protein-lipid-ion association. One of the most important problems in membrane biophysics is how the membrane components associate to form a stable membrane. In order to study this association, vesicles could be formed from phospholipid, a

membrane protein which contains sulfur, and various ions. If the components are properly mixed, they will form membranes which can be shown to have certain physiological functions and can be separated from the medium by centrifugation.⁷¹ Targets could be formed from these vesicles and analyzed by PIXE. The amount of each component can be determined, since the phospholipid content is given by the amount of phosphorus, the protein component is given by the amount of sulfur, and the ionic content is determined directly. Available components could be varied among the phospholipids, numerous membrane proteins which have been extracted, and ions which are of interest in membrane formation and stability. This type of study could yield valuable information concerning the types of association required for membrane formation and stability.

APPENDIX A

ENERGY LOSS OF PROTONS IN BIOLOGICAL SAMPLES

The stopping cross section can be written as:

$$\frac{dE}{dx} = -N\epsilon$$

where $\frac{dE}{dx}$ = energy loss per unit distance

N = number of stopping atoms per unit volume

and ϵ = stopping cross section per atom.⁷²

For oxygen an empirical curve for ϵ is given by

$$\epsilon = (1.92/E_p) \left[\ln \left(\frac{E_p}{8} \right) + 5.12 \right] 10^{-15} \text{ ev-cm}^2/\text{atom}$$

where E_p = proton energy in MeV.

For 5 MeV protons $\epsilon = 1.79 \times 10^{-15} \text{ ev-cm}^2/\text{atom}$.

If a density of one gram per cubic centimeter is used for biological samples then for oxygen, $N \approx 3.75 \times 10^{22}$ atoms per cubic centimeter. Then

$$\frac{dE}{dx} = -67 \text{ MeV/cm}.$$

A biological target .1 milligrams per square centimeter would be 10^{-4} centimeters thick and the energy loss in traversing this target would be .0067 MeV.

If a more realistic composition were assumed, including carbon and hydrogen, ϵ would be slightly lower. Thus, it is concluded that the change in production cross section can be ignored in this study.

APPENDIX B

COLLECTION OF BOVINE RETINAS

Within 20 minutes after the animal is slaughtered the whole eyes are removed and placed in a container of wet ice. After 50 are collected they are taken to a darkroom where the retinas are removed under red light. Each retina is then placed in a vial which is on dry ice, rapidly freezing the retina. The vials are filled with 50 retinas, wrapped in aluminum foil and transferred to freezer storage. The collection of 50 eyes takes approximately 15 minutes as does the removal of 50 retinas (John Schmitz, Hormel and Co., Austin, Minnesota, private communication).

APPENDIX C

SEPARATION PROCEDURE FOR INTACT DISC MEMBRANES

Fifty bovine retinas were defrosted and shaken for three-five minutes in 45% sucrose, .1 Molar (M) phosphate buffer (pH 7). The suspension was equally divided and spun at 25,000 revolutions per minute (rpm) in an SW-27 rotor. The float material from each tube was pipetted into a Sorvall centrifuge tube. The tubes were filled with 1 M-phosphate buffer and spun in a Sorvall SS-34 rotor for 20 minutes at 15,000 rpm. The supernatant was decanted from each tube and the pellet suspended in 40% sucrose, 1 M-phosphate buffer. This was layered with .1 M-phosphate buffer and spun at 25,000 rpm for one hour in an SW-27 rotor. The isolated rod outer segments were pipetted from the interface. They were then placed in double distilled water in a Sorvall tube and spun at 17,500 rpm for 30 minutes. The resulting pellet was suspended in 5% Ficoll and left for 10-14 hours in order to burst the rod plasma membrane. They were then spun in an SW-27 rotor for two hours at 25,000 rpm. The intact discs were pipetted from the surface and spun twice in 5% sucrose at 17,500 rpm for 30 minutes in a Sorvall tube.⁶⁴ All centrifugations in Sorvall tubes were done in an SS-34 rotor.

APPENDIX D

ESTIMATION OF BLEACHING LEVELS

Bleaching was done with a standard 40 watt bulb rated at 455 lumens. At 555 nanometers, one lumen equals 5.72×10^{-4} watts⁷³ which equals 1.47×10^{15} quanta per second. Therefore, a 40 watt bulb yields 6.69×10^{17} quanta per second. At one meter distance the quantal flux is 5.32×10^{12} quanta per square centimeter per second.

Rhodopsin has a molecular extinction coefficient of 1.56×10^{-20} square meters.⁷⁴ Ignoring screening effects the ratio of rhodopsin bleached per second at one meter will be .00083. A one meter distance was used for low bleaching levels. When higher levels were desired (above 1%) the samples were placed closer to the light. Samples which were 100% bleached were exposed to room lights for a minimum of ten minutes and no coloration was observable. In addition to screening, the absorption of the plastic vials was ignored.

APPENDIX E

HEX

HEX is a Fortran program specifically designed for analysis of PIXE spectra. It is a non-linear least squares fit to the data utilizing numerous parameters. Some are determined previously and held fixed during the fit, and others are allowed to vary. However, most of the free parameters represent physical parameters obtained from models of the data acquisition process.

The background model combines both Compton scattering and bremsstrahlung. The parameters dealing with Compton scattering are empirical and determined during the fit. The fitting function for Compton scattering is the sum of a constant and a slowly decaying exponential. The bremsstrahlung model was determined from the physical processes and these parameters have physical significance. It is modeled by a decreasing exponential.

The peaks are fitted with modified Gaussians which reflect the loss of detector resolution as X-ray energy increases. Each element in the library is given a fixed K_α to K_β intensity ratio which has been experimentally determined. Thus one linear parameter is used to represent each element detected in the sample.

The remaining parameters treat the absorption problem. The absorption external to the target is calculated from X-ray absorption coefficients of the material through which the X-rays must pass after leaving the target. For each system configuration a transmission curve is calculated. This curve is then held fixed during each PIXE fit. As previously mentioned, a unique feature of HEX is its ability to calculate and correct for self-absorption. This is done with an exponential containing two parameters which are determined in the least squares fit and can be related to the composition and thickness of the target.

All parameters are determined simultaneously during the Marquardt least squares fit and the linear parameters modifying the Gaussians' heights are then used to determine the amount of the appropriate element. HEX incorporates system efficiency values determined by bombarding commercial standards. These are guaranteed to 5% accuracy.

Error analysis is done by HEX allowing 5% error for standards and 2% for electronics. In addition, the fitting

error is included. It is also determined by HEX from the error matrix.

Part of the input data to HEX is the percentages of light elements in the matrix. This is held constant and used to calculate self absorption, consistent with the parameter for thickness, in the fitting procedure. Since the composition of the matrix was not known it was estimated from known protein and lipid compositions. These values were checked by increasing the oxygen content (oxygen was the heaviest element in the matrix) until a total mass equal to the measured mass of the target was obtained. The other extreme is known because it would be a matrix with no absorption. The differences between absorptions calculated at these two extremes was typically 20%. Assuming a 95% certainty leads to a 5% error in choice of matrix composition. This was combined with the error determined by HEX for sulfur. The ratio of each detected element to sulfur was computed and the errors combined as independent errors.

It is obvious that some of the errors associated with fitting, standardization, electronics and absorption must cancel if the ratio of two elements is used. This is because the errors are not independent and should have been combined differently. However, a realistic calculation of the interdependence was not possible and errors are as indicated. Thus, it is felt that the errors reported are probably too large.

It should be noted that HEX does not consider silicon escape peaks nor X-ray absorption edges. The effect of these omissions is thought to be very small; however, it is not possible to determine if this is true without extensive experimentation. Regardless of this, it is felt that HEX is the best data reducing program in use in PIXE work.

APPENDIX F

MITOCHONDRIA ASSAY

The assay used measures the change in absorbance at 455 nanometers due to the reduction of $K_3Fe(CN)_6$ by succinate dehydrogenase. The change in absorbance is then related to the succinate dehydrogenase activity through the known molar extinction coefficient.⁷⁵

The method of Bradford was used to determine the protein concentration.⁷⁶ This enabled the succinate dehydrogenase activity to be determined per milligram of protein. The result for crude rod outer segment preparation was 5.6 micromoles/minute/milligram of protein.

APPENDIX G

ESTIMATION OF UPPER LIMIT FOR THE AMOUNT OF Ba PRESENT IN DISC MEMBRANES

The L lines of Ba which have energies between 4.451 and 5.995 KeV lie between the K lines of Ca and Fe. This region of the spectra appeared void of peaks indicating no Ba was detected. However, if Ba has a role in the visual process it should be present in detectable amounts. Therefore, it is of interest to determine an upper limit on the amount of Ba present.

Using Figure 4, it can be seen that the detection concentrations for Z=56 (using the L line curves) are approximately five times higher than Z=24 (chromium, using K line curves). Since the computer code HEX yields a minimum detection limit for each element contained in the library, these two can be combined to yield the desired Ba estimate. The minimum detection limit for chromium was typically two nanograms per square centimeter implying a limit for Ba of ten nanograms per square centimeter. Using sulfur values as 3×10^3 nanograms per square centimeter yields an upper limit of .02 Ba per rhodopsin.

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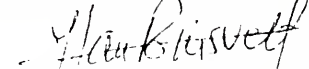
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BIOGRAPHICAL SKETCH

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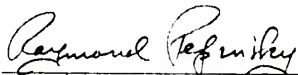
Henri A. Van Rinsvelt, Chairman
Professor of Physics

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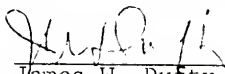
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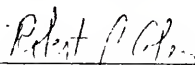
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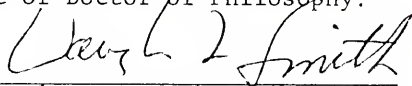
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